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Candida guilliermondii Complex Is Characterized by High Antifungal Resistance but Low Mortality in 22 Cases of Candidemia

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ABSTRACT The objectives of our study were to describe the characteristics of patients with *Candida guilliermondii* candidemia and to perform an in-depth microbiological characterization of isolates and compare them with those of patients with *C. albicans* candidemia. We described the risk factors and outcomes of 22 patients with candidemia caused by the *C. guilliermondii* complex. Incident isolates were identified using molecular techniques, and susceptibility to fluconazole, anidulafungin, and micafungin was studied. Biofilm formation was measured using the crystal violet assay (biomass production) and the XTT reduction assay (metabolic activity), and virulence was studied using the *Galleria mellonella* model. Biofilm formation was compared with that observed for *C. albicans*. The main conditions predisposing to infection were malignancy (68%), immunosuppressive therapy (59%), and neutropenia (18%). Clinical presentation of candidemia was less severe in patients infected by the *C. guilliermondii* complex than in patients infected by *C. albicans*, and 30-day mortality was lower in *C. guilliermondii* patients (13.6% versus 33.9%, respectively; $P = 0.049$). Isolates were identified as *C. guilliermondii sensu stricto* ($n = 17$) and *Candida fermentati* ($n = 5$). The isolates produced biofilms with low metabolic activity and moderate biomass. The *G. mellonella* model showed that *C. guilliermondii* was less virulent than *C. albicans* (mean of 6 days versus 1 day of survival, respectively; $P < 0.001$). Patients with candidemia caused by the *C. guilliermondii* complex had severe and debilitating underlying conditions. Overall, the isolates showed diminished susceptibility to fluconazole and echinocandins, although poor biofilm formation and the low virulence were associated with a favorable outcome.

KEYWORDS *Candida guilliermondii*, *Candida fermentati*, *Galleria mellonella*, non-*albicans* candidemia, biofilm formation

Candida albicans is the main cause of candidemia, although a shift toward other non-*albicans Candida* species, such as *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*, has been reported (1–4). The remaining infrequent *Candida* species account for 8% of episodes (5); however, their epidemiological characteristics remain largely unknown.

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C. guilliermondii is a complex comprising several species, of which 3 have been reported as human pathogens (*C. guilliermondii sensu stricto* [*Meyerozyma guilliermondii* as the teleomorph form], *C. fermentati* [*M. caribbica* as the teleomorph form], and *C. carpophila*). The proportion of candidemia episodes caused by the *C. guilliermondii* complex has been poorly studied, although it is thought that this species is involved in up to 2% of cases (3, 6–9). The *C. guilliermondii* complex is characterized by diminished susceptibility to azoles and echinocandins (9, 10) and has been reported mostly as a cause of candidemia in patients with cancer (11, 12). However, the low number of cases reported means that knowledge of the epidemiology of candidemia caused by this complex is limited (9, 11).

We report 22 cases of candidemia caused by the *C. guilliermondii* complex and compare them with cases of candidemia caused by *C. albicans* to identify differential characteristics. We also performed in-depth microbiological characterization of *C. guilliermondii* isolates based on molecular identification of species, antifungal susceptibility, biofilm formation, and virulence.

(This study was presented in part at the 26th European Congress on Clinical Microbiology and Infectious Diseases in Amsterdam [Abstr P-0350] [13].)

RESULTS

Patient demographics, risk factors, and outcome. Episodes caused by *C. albicans* in the CANDIPOP study were compared with the overall cohort of *C. guilliermondii* episodes to identify differential characteristics. To preserve the assumption of independence of observations, only the first episode of candidemia recorded for an individual patient was included in this analysis, and episodes caused simultaneously by different *Candida* species were excluded. Therefore, 22 *C. guilliermondii* episodes were finally compared with 337 *C. albicans* episodes (Table 1). A univariate analysis showed that patients infected by *C. guilliermondii* were more likely to have underlying malignancy or neutropenia or to have undergone solid organ transplantation. Overall, 59% of these patients had received immunosuppressive therapy within the previous month. Previous exposure to antifungals and long-term central venous catheters (CVCs) were also more common in this group. Conversely, patients with *C. albicans* infection were older, had more comorbidities (e.g., diabetes), and were more likely to receive total parenteral nutrition.

Clinical data, therapeutic measures, and outcomes. The clinical presentation of candidemia was less severe in patients infected by *C. guilliermondii* than in those infected by *C. albicans*. None of the patients infected by *C. guilliermondii* had septic shock or severe sepsis at the onset of candidemia. Moreover, the *C. guilliermondii* infections were less likely to occur during hospitalization in the intensive care unit, and 27.3% of cases were outpatient acquired, with all but one being health care related (Table 1). The therapeutic measures are shown in Table 2. Briefly, early removal of the CVC was less frequent in patients infected by *C. guilliermondii* than in those infected by *C. albicans* (15.8% versus 50.2%, respectively; $P = 0.004$). However, no differences were seen between patients infected by *C. guilliermondii* and those infected by *C. albicans* with respect to the frequency of catheter-related candidemia (27.3% versus 30.3%, respectively). Early appropriate antifungal treatment was less common in patients infected by *C. guilliermondii* than in those infected by *C. albicans* (36.4% versus 60.5%, respectively; $P = 0.026$), mainly because therapy was started more than 48 h after diagnosis (8/14 [57.1%]) or because of empirical treatment with fluconazole (5/14 [35.7%]).

As for prognosis, persistent candidemia (blood cultures that were persistently positive for $\geq$3 days after the incident blood sample) tended to be more common in patients infected by *C. guilliermondii* than in those infected by *C. albicans* (38.5% versus 26%, respectively). However, the 30-day mortality was lower in the *C. guilliermondii* group than in the *C. albicans* group (13.6% versus 33.9%, respectively; $P = 0.049$).

Microbiological characterization of the isolates. All cases were caused by *C. guilliermondii sensu stricto* ($n = 17$) or *C. fermentati* ($n = 5$). In terms of biofilm

TABLE 1 Comparison of baseline characteristics and clinical data of patients with *C. guilliermondii* and *C. albicans* candidemia

Variable[a]	*C. guilliermondii* (*n* = 22)	*C. albicans* (*n* = 337)	*P* value
Demographic			
Median age (years) (IQR)	50.9 (10.0–67.7)	65.2 (45.4–75.8)	0.040
Age ≥65 years (*n* [%])	6 (27.3)	171 (50.7)	0.033
Male sex (*n* [%])	12 (54.5)	185 (54.9)	0.974
Outpatient (*n* [%])	6 (27.3)	31 (9.2)	0.017
In ICU at diagnosis[b] (*n*/total [%])	2/16 (12.5)	131/306 (42.8)	0.016
Days in hospital until *Candida* BSI[b] (IQR)	29.0 (13.3–66.5)	19 (11–34)	0.079
Comorbidity (*n* [%])			
Diabetes mellitus	0	76 (22.6)	0.006
Malignancy	15 (68.2)	102 (30.3)	<0.001
Hematological malignancy	6 (27.3)	13 (3.9)	<0.001
Chronic renal failure	4 (18.2)	43 (12.8)	0.509
Solid organ transplant	2 (9.1)	11 (3.3)	0.186
Liver cirrhosis	1 (4.5)	13 (3.9)	0.594
HIV infection	1 (4.5)	6 (1.8)	0.360
Risk factor for candidemia (*n* [%])			
CVC	19 (86.4)	258 (76.6)	0.288
Long-term CVC	7/19 (36.8)	30/258 (11.6)	0.007
Total parenteral nutrition	7 (31.8)	176 (52.2)	0.064
Immunosuppressive therapy[c]	13 (59.1)	68 (20.2)	<0.001
Neutropenia (<500 cells/mm^3)	4 (18.2)	10 (3)	0.007
Abdominal surgery (3 mo)	4 (18.2)	95 (28.2)	0.309
Prior fungal therapy[c]	7 (31.8)	53 (15.7)	0.071
Azole exposure	5 (22.7)	37 (11)	0.159
Echinocandin exposure	0 (-)	9 (2.7)	1.000
Breakthrough candidemia	4 (18.2)	27 (8)	0.110
Source of infection (*n* [%])			
Primary	15 (68.2)	201 (59.6)	0.428
Catheter related	6 (27.3)	102 (30.3)	0.767
Urologic	0	22 (6.5)	0.382
Abdominal	0	9 (2.7)	1
Other	1 (4.5)	3 (0.9)	0.224
Severity of infection (*n* [%])			
Septic shock or severe sepsis	0	120 (35.6)	0.001

[a]ICU, intensive care unit; BSI, bloodstream infection; CVC, central venous catheter; HIV, human immunodeficiency virus.
[b]Only includes nosocomial candidemia or cases with positive blood culture after 2 days of hospitalization.
[c]Within the preceding month. Immunosuppressive therapy includes corticosteroids, chemotherapy, and other immunosuppressive drugs.

formation, the isolates were moderate biofilm forming ($n = 13$) or low biofilm forming ($n = 9$), with moderate metabolic activity ($n = 9$), low metabolic activity ($n = 12$), or high metabolic activity ($n = 1$). Scanning electron microscopy revealed that the *C. guilliermondii* complex biofilms were formed mainly by a layer of clamped blastospores without hyphae or pseudohyphae, as seen in cornmeal agar medium (Fig. 1). Table 3 shows the antifungal susceptibility of the isolates in both the planktonic and sessile forms. Up to 10% (2/22) of the isolates had fluconazole MICs above the epidemiological cutoff values (ECOFFs). Differences in susceptibility to all the drugs tested between *C. albicans* and *C. guilliermondii* complex isolates were observed in the planktonic form ($P < 0.001$). According to the susceptibility of the biofilms, no differences reaching statistical significance were observed between anidulafungin and micafungin (sessile MIC_{50} [$SMIC_{50}$] geometric mean of 2.46 μg/ml and 8.28 μg/ml, respectively; $P = 0.096$).

C. guilliermondii complex isolates were significantly less virulent than *C. albicans* isolates (6 versus 1 mean survival days; $P < 0.001$) in the *Galleria mellonella* model (Fig. 2). *C. fermentati* isolates were more virulent than *C. guilliermondii sensu stricto* isolates

TABLE 2 Therapeutic measures and outcomes of patients infected by *C. guilliermondii* complex and *C. albicans*

Agent, measure, or outcome	No. (%) of *C. guilliermondii* isolates ($n = 22$)	No. (%) of *C. albicans* isolates ($n = 337$)	*P* value
Initial antifungal agent			
Azole	11 (50)	171 (50.7)	0.946
Echinocandin	4 (18.2)	85 (25.2)	0.459
Amphotericin B	5 (22.7)	48 (14.2)	0.346
Combination of drugs	1 (4.5)	4 (1.2)	0.272
Therapeutic measures (≤48 h)			
CVC removal[a]	3/19 (15.8)	127/253 (50.2)	0.004
Follow-up blood cultures[b]	19/22 (86.4)	219/337 (65)	0.040
Persistent candidemia[c]	8/19 (42.1)	57/219 (26)	0.131
Causes of inadequate therapy ($n = 148$)			
Initiation beyond first 48 h	8/14 (57.1)	82/134 (61.2)	
Suboptimal fluconazole doses[d]	5/14 (35.7)	23/134 (17.2)	
No targeted antifungal treatment	1/14 (7.1)	29/134 (21.6)	
Clinical response and outcome			
Disseminated infection[e]	4 (18.2)	33 (9.8)	0.265
Ocular candidiasis	3 (13.6)	16 (4.7)	0.102
Endocarditis	1 (4.5)	9 (2.7)	0.473
Central nervous system	0	7 (2.1)	1.000
Other	1 (4.5)[f]	3 (0.9)[f]	0.224
7-day mortality	1 (4.5)	43 (12.8)	0.497
14-day mortality[g]	2 (9.1)	72/335 (21.5)	0.274
30-day mortality[g]	3 (13.6)	113/333 (33.9)	0.049

[a]In the subset of adult patients with central venous catheters (CVCs; $n = 277$). Data regarding CVC removal in the first 48 h were missing in 5 cases in the *C. albicans* group.
[b]Extraction of follow-up blood cultures ≥3 days after the incident blood culture.
[c]Analysis performed in the subset of patients with follow-up blood cultures ≥3 days after incident blood culture.
[d]Fluconazole at <400 mg/day for susceptible *Candida* isolates or at any dose for *C. guilliermondii* isolates.
[e]Three patients had ≥1 affected organ.
[f]Includes 1 metastatic kidney infection, 1 metastatic lung infection, and 2 cases of cutaneous dissemination (one in an extremely low-birth-weight preterm infant and the other in a neutropenic patient).
[g]Four patients were lost to follow-up before day 30 (2 before day 14) in the *C. albicans* group.

(4 versus 9.5 mean survival days; $P < 0.001$), although the low number of isolates of this species limited the analysis. We did not find differences in terms of biofilm formation or susceptibility between *C. fermentati* and *C. guilliermondii sensu stricto* isolates (data not shown).

DISCUSSION

The *C. guilliermondii* complex comprises *C. guilliermondii sensu stricto*, *C. fermentati*, *C. carpophila*, and other species (6, 7). In previous studies, *C. guilliermondii sensu stricto* and *C. fermentati* accounted for approximately 90% to 95% and 4.5% to 9% of cases, respectively, with the remaining species of the complex playing a marginal role (less than 1%) (9, 14). By contrast, we found a higher frequency of cases caused by *C. fermentati* (22.7%) and a lower frequency of cases caused by *C. guilliermondii sensu stricto* (77.2%).

The complex exhibited decreased *in vitro* susceptibility to all of the antifungal agents studied, with rates of resistance to fluconazole and other azoles of approximately 10% to 15% (9, 12, 14). Up to 10% of the isolates studied here were non-wild type according to the fluconazole ECOFF. In the absence of species-specific breakpoints for *C. guilliermondii*, the rate of true fluconazole resistance remains unknown. The MICs of micafungin and anidulafungin against *C. guilliermondii* were 16- and 60-fold higher, respectively, than the MICs against *C. albicans* reported in the literature (3, 15). We also observed that micafungin and anidulafungin were similarly effective at causing damage to the *C. guilliermondii* biofilms, although the clinical impact of this observation is unknown.

Candidemia caused by *C. guilliermondii* commonly originates in the CVC (11, 16). We

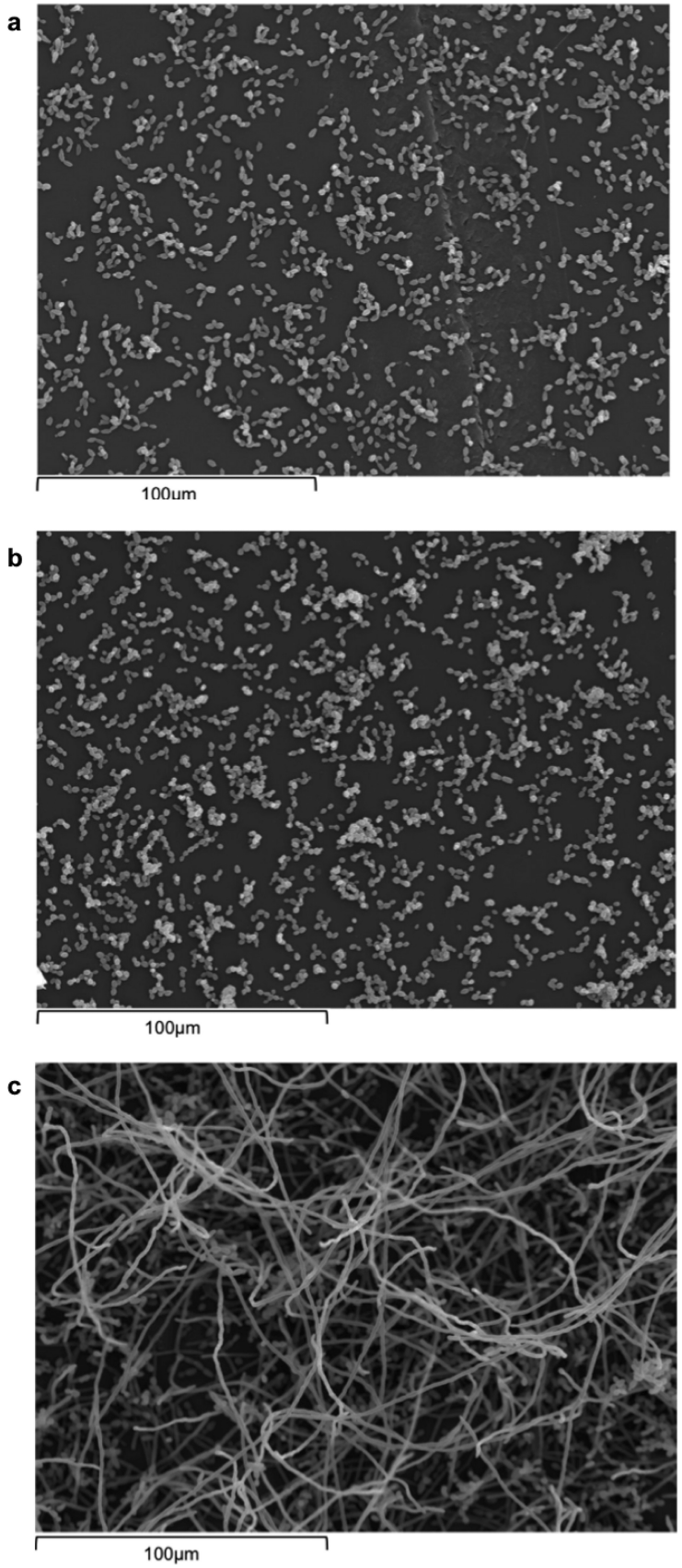

FIG 1 Biofilms formed by *C. fermentati* (a), *C. guilliermondii sensu stricto* (b), and *C. albicans* (c) isolates. ×500 magnification.

TABLE 3 Antifungal susceptibilities of *C. guilliermondii* complex and *C. albicans* isolates from the CANDIPOP study[a]

	MIC (μg/ml)								
	C. guilliermondii complex biofilm $SMIC_{50}$ (μg/ml)			*C. guilliermondii* complex (n = 22)			*C. albicans* (n = 337)		
Antifungal	GM	MIC_{90}	Range	GM	MIC_{90}	Range	GM	MIC_{90}	Range
Micafungin	8.28	≥512	0.25 to ≥512	0.27	1	0.25–4	0.03	0.031	0.031–1
Anidulafungin	2.46	232	0.062 to ≥512	1	2	0.5–1	0.03	0.031	0.031–0.25
Fluconazole	NT			5.27	16	0.5 to ≥64	0.21	0.25	≤0.125 to ≥64
Posaconazole	NT			0.08	0.5	0.031–1	0.015	0.015	≤0.015 to ≥8
Voriconazole	NT			0.10	0.5	≤0.015–2	0.016	0.015	≤0.015 to ≥8
Amphotericin B	NT			0.31	1	0.062–0.5	0.05	0.125	0.031–0.25

[a]See reference 3. $SMIC_{50}$, sessile MIC corresponding to 50% reduction of biofilm metabolic activity; GM, geometric mean; NT, not tested.

found that CVCs were involved in a high percentage (86.4%) of patients, as was the case in patients infected by *C. albicans*. However, *C. guilliermondii* strains developed biofilms with moderate or low biomass and were significantly different from *C. albicans* isolates (Fig. 1). This apparent disagreement between the CVC as the source of infection and the scant ability to form biofilms may be due to the fact that many cases of CVC-related candidemia involved long-term CVCs (36.8%), unlike those infected by *C. albicans* (11.6%). Moreover, the method chosen to study the biomass or metabolic activity implied shorter biofilm maturity (24 h).

C. guilliermondii has been reported to be a cause of infection, mainly in patients with solid tumors and hematological malignancies, patients undergoing chemotherapy, patients with neutropenia, patients with intravascular and vascular catheters, patients receiving total parental nutrition, and patients with previous exposure to antibiotics, corticosteroids, and long-term antifungal therapy (mostly fluconazole, amphotericin B, and caspofungin) (11, 12, 17). We found that patients with candidemia caused by *C. guilliermondii* were more likely to have solid cancer or hematological malignancies, thus supporting some of the previously reported risk factors associated with this infection.

As for the prognosis, it seems reasonable to expect mortality to be high, considering the severe underlying conditions of the patients and the low susceptibility of the isolates to the antifungal drugs. However, we found that 7-day and 30-day mortalities were lower and clinical presentations were less severe in patients infected by *C. guilliermondii* than in patients infected by *C. albicans*. This observation is in agreement with the low virulence of *C. guilliermondii* isolates compared with that of *C. albicans*, as shown in the *G. mellonella* model ($P < 0.001$). Although this model has been used to study the virulence of several *Candida* species (18), to our knowledge, this is the first time it has been used for *C. guilliermondii*. The low virulence of the isolates can also be explained by the limited production of biofilm, as previous studies showed that intense biofilm formation is an independent factor of poor prognosis (19). The *G. mellonella* model is relatively easy to handle. The correlation between the favorable prognosis of

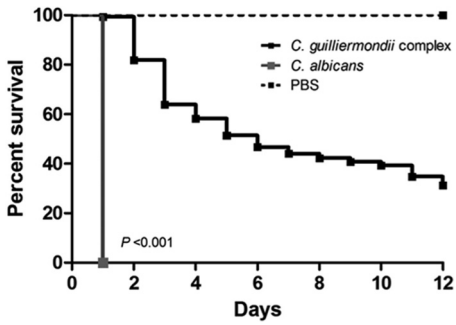

FIG 2 Survival curves of *G. mellonella* infected with the *C. guilliermondii* complex and *C. albicans* isolates (of 5×10^6 yeast cells/larva) after 12 days of incubation at 37°C.

the patients and the high survival of the larvae is encouraging and suggests that this model could be used in the clinical microbiology laboratory to screen for the presence of highly virulent isolates.

In conclusion, the *C. guilliermondii* complex mainly affected patients with severe debilitating conditions. The isolates were characterized by moderate biofilm production, low susceptibility to azoles and echinocandins, and low virulence. However, the prognoses of the patients infected by this species are favorable.

MATERIALS AND METHODS

Patients and isolates. We described the risk factors and outcome of 22 patients with episodes of monomicrobial candidemia caused by the *C. guilliermondii* complex who were admitted to Gregorio Marañon Hospital, Madrid ($n = 9$), or Vall d'Hebron Hospital, Barcelona ($n = 5$), from 2007 to 2014. We also included cases collected in the remaining hospitals participating in the CANDIPOP project ($n = 8$), a multicenter population-based study on candidemia conducted from 2010 to 2011 in Spain (3, 20). The incident isolates were identified using amplification and sequencing of the ITS1-5.8S-ITS2 region (3, 21).

Data collection. Cases were identified from the microbiological database of each participating hospital. Data from the CANDIPOP study were collected prospectively, as previously described (3, 20). For cases that were not included in the CANDIPOP study, clinical records were collected from Gregorio Marañon Hospital and Vall d'Hebron Hospital.

Definitions. The definitions used have been reported elsewhere (20, 22). In summary, episodes detected at or within 2 days of hospital admission were considered outpatient acquired. These episodes could also be classified as health care related if patients fulfilled any of the following criteria: hospitalization for ≥2 days within the 90 days prior to candidemia, residence in a long-term-care facility, or chemotherapy or dialysis in the 30 days before diagnosis of candidemia (23). The criteria for proven catheter-related candidemia were those set out in the Infectious Diseases Society of America (IDSA) guidelines (24). Secondary foci required isolation of the same *Candida* species in the suspected source of infection. Breakthrough episodes were defined as candidemia in patients who had been receiving antifungal drugs for >3 days. The presence of severe sepsis or septic shock was recorded on the day candidemia was diagnosed (25, 26). Adequate early antifungal treatment was defined as the correct dose of antifungal agent for a susceptible *Candida* isolate administered within the first 48 h after the blood sample collection. Early CVC removal was defined as within the first 48 h after the incident positive blood culture. In patients with multiple CVCs, at least the responsible CVC had to be removed within this time frame.

Antifungal susceptibility testing. The antifungal susceptibility of the planktonic isolates was tested against the following drugs: amphotericin B (Sigma-Aldrich, Madrid, Spain); fluconazole, voriconazole, and anidulafungin (Pfizer Pharmaceutical Group, New York, NY, USA); posaconazole (Merck & Co., Inc., Rahway, NJ, USA); and micafungin (Astellas Pharma, Inc., Tokyo, Japan). Due to the lack of interlaboratory reproducibility, caspofungin was not studied (27).

The procedure used was that of EUCAST EDef 7.2 (28). The antifungal agents were tested at concentrations ranging from 0.015 to 8 μg/ml, except for fluconazole, which was tested at 0.062 to 64 μg/ml. MIC values were determined spectrophotometrically at 530 nm (Multiskan FC microplate photometer; Thermo Scientific, Madrid, Spain) after 24 h of incubation and were defined as the lowest concentration of drug that resulted in inhibition of ≥50% of growth in comparison with a drug-free growth control well for fluconazole, voriconazole, posaconazole, and echinocandins or inhibition of ≥90% for amphotericin B. According to the *C. guilliermondii* complex ECOFFs, previously reported isolates were considered non-wild type to fluconazole if they had an MIC >16 μg/ml (29).

Biofilm formation and antifungal susceptibility. We studied biofilm formation using crystal violet staining and by measuring the metabolic activity of the biofilm using the XTT reduction assay. We classified the isolates according to their biomass formation and metabolic activity based on our previously reported score (30) as follows: low, moderate, and high biofilm forming (<0.44, 0.44 to 1.17, and >1.17, respectively), and low, moderate, and high metabolic activity (<0.097, 0.097 to 0.2, and >0.2, respectively). The antifungal activity of anidulafungin and micafungin against sessile cells was assessed using the XTT reduction assay at concentrations ranging from 0.015 μg/ml to 512 μg/ml. The sessile MIC_{50} ($SMIC_{50}$) was defined as a 50% reduction in the metabolic activity of the biofilm treated with the drug compared with that of the drug-free control well (31).

Scanning electron microscopy. We studied the structures of the biofilms formed by three isolates: *C. guilliermondii sensu stricto* ($n = 1$), *C. fermentati* ($n = 1$), and *C. albicans* ($n = 1$). Biofilms were formed on 50-mm polystyrene discs and prepared for scanning electron microscopy as previously reported (30). The structures of the biofilms were visualized using a scanning electron microscope (JEOL-JSM 6400; Jeol, Tokyo, Japan).

Study of virulence with the *Galleria mellonella* model. We studied virulence using final-instar larvae of *Galleria mellonella* (Bichosa, Salceda de Caselas, Spain). Inocula were standardized to enable survival of 50% of the larvae 96 h after infection with 4 strains (5×10^8 yeast cells/ml [5×10^6 yeast cells/larva]). Briefly, inocula were prepared after an overnight incubation of the isolates in yeast extract-peptone-dextrose; the inocula were then washed twice with phosphate-buffered saline (PBS) and adjusted using an automatic cytometer (Moxi flow cytometer; Orflo Technologies, Ketchum, ID, USA). *G. mellonella* larvae were injected directly into the hemocoel via the last left proleg using a Hamilton syringe

and were incubated at 37°C in plastic petri dishes. Dead larvae were counted daily for 12 days. Each experimental group contained 16 randomly chosen larvae of an appropriate weight (330 ± 20 mg). Three control groups containing the same numbers of larvae were included to monitor the insult (PBS was used for injection), the survival of noninjected larvae, and the virulence of 6 randomly chosen *C. albicans* isolates (5×10^8 yeast cells/ml [5×10^6 yeast cells/larva]).

Data analysis. Quantitative variables are reported as medians and interquartile ranges (IQRs) and qualitative variables are reported as counts (percentages). Categorical variables were compared using the chi-square or Fisher exact tests, as appropriate, and continuous variables were compared with the Mann-Whitney U test. Statistical analyses were performed using Microsoft SPSS-PC+, version 15.0 (SPSS, Chicago, Illinois, USA). *P* values of < 0.05 were considered significant. The activity of the antifungal drugs is shown as the geometric mean MIC, MIC_{90}, and range of MICs for planktonic and sessile forms; drug activity was compared using the Friedman test. Killing curves were analyzed using the Kaplan-Meier method with GraphPad Prism 5.02 software (GraphPad, La Jolla, CA, USA). Differences in median survival between species were evaluated using the Mann-Whitney U test.

Ethical considerations. This study (protocol no. 39/15) was approved by the ethics committee of Hospital Gregorio Marañón (CEIC-A1). The need for informed consent was waived owing to the retrospective design of the study. The CANDIPOP study was approved by the local institutional review boards, and written consent was obtained from all patients.

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